RECENT INNOVATIONS IN THE FABRICATION OF TISSUE ENGINEERED IMPLANTABLE CONSTRUCTS FOR TENDON/LIGAMENT REGENERATION

Abstract

Tendons and ligaments are being frequently damaged and have a low regenerative capability due to their physiology and anatomy. Restoration of the properties of the abovementioned tissues cannot be achieved by the current therapeutic procedures. Fortunately, developed tissue engineered constructs showed better results while being assessed in vitro and in vivo to heal damaged tendons and ligaments. Herein, recent innovations in scaffold fabrication, cell loading procedures and biochemomechanical stimulations and their results are expressed and investigated.

Keywords: tendon, ligament, regeneration, tissue engineering, scaffold, cell loading, biomechanical stimulation, chemical stimulation, noninvasive imaging

Introduction

Ligaments and tendons are special fibrous tissues and essential parts of our musculoskeletal system.[1] Tendon tissues structurally connect bones to muscles while mechanically alleviating stress from one side to the other.[2] Ligaments on the other hand, structurally link bones to bones while mechanically controlling the stability of the movement.[2] There are over 30 million musculoskeletal injuries reported annually and over 50% of these are due to tendons and ligament defects.[3] The elderly population is predominantly prone to tendon injuries.[3] Also, the population of athletes with high mechanical activity and weight-bearing is always at risk of tendon rupture.[1,3–7] The process of healing is hard and slow due to the hypo-vascularity and low cellularity of the mentioned tissues leading to the limited amount of oxygen and nutrients necessary for cells to survive.[1,6,8–10] Usually healing does not result in biomechanical restoration due to fibrovascular scar tissue formation instead of fibrocartilage regeneration resulting in retears and insufficient biomechanical in comparison to those of the native tissue properties.[4,6,7,11–13] Current methods to treat these injuries include autografts, allografts and xenografts for large defects.[1,3,6,7,10,14] Application of autografts is limited due to the normal healthy tissue utilization, prolonged surgical time, limited amount of harvested tissue and donor site morbidity.[1,6,7,9,15,16] Allografts and xenografts may result in foreign body reaction, transmission and undesired immune responses.[1,6,9,12,14,16] It is worth mentioning that the risk of postsurgical complications and retears still exists.[10] Due to these limitations of these theraputic techniques tissue engineered scaffolds have been developed to achieve better mechanical, biological and structural restoration.[9,10,12,14] The ideal scaffold possesses: (1) pore size distribution optimized for promotion of cell migration; (2) sufficient surface area with biochemical properties that encourage cell adhesion, proliferation, migration and differentiation; and (3) degradation rate matched with regeneration rate of the targeted tissue.[16]

Table 1: Reported tensile strength, modulus, water content and collagen content of selected T/L.[19]

T/L tissue	Tensile strength [MPa]	Tensile modulus [MPa]	Water content [% of wet mass]	Collagen content [% of dry mass]
AT	86 ± 24[7] 54 ± 20[16]	822 ± 211[15] 212 ± 109[16] 670–1070[17]	69.0 ± 2.9%[14] 66.2 ± 5.5%[18]	77.2 ± 1.3%[14] 64.5 ± 4.6%[18]
RCT/SST	4–16[19] 8–24[20]	10–170[21] 50–170[19] 168[22]	75.1 ± 3.9[23]	66.6 ± 5.3[23]
ACL	24 ± 9[24] 13 ± 5[old][2] 38 ± 9[young][2]	113 ± 45[24] 65 ± 24[old][2] 111 ± 26[young][2]	~67%[21]	~75%[21]

Tendon and ligament

Collagen type I and III fibrils with a diameter of 35–500 nm, arrange to form bundles in the tendon or ligament.[17,18] Several bundles form fascicles and fascicles organize to form a tendon or ligament.[17,18] The alignment of collagen fibrils in the tendon and ligaments is exclusively unidirectional and longitudinally oriented between muscle and bone supplying tensile strength in this direction.[17,18] Despite all the similarities of tendon and ligament tissue structures, some structural differences exist.[17,18] Tendon fibers are mainly organized along the main axis while ligament fibers are more randomly oriented, with a weaving pattern. Tendons are slightly higher in collagen content[2] and also include slightly lower ground substances such as hyaluronan, glycoproteins, proteoglycans and elastin rather than ligaments. Therefore, at the regarding these differences in structure and ECM composition, the tensile strength and elastic modulus are higher in tendons rather than ligaments.[2,3,19]

Scaffold fabrication methods

Development of a living graft replacing the fixation device can solve some of the problems of surgical repairs.[4] Recapitulation of the structural and mechanical properties of the ECM of the target tissue is necessary and hard due to these properties changing significantly from one part of the body to another.[11,14]

Biomaterials

These scaffolds made of natural or synthetic polymers and hybrid designs consisting of native and manufactured materials seem to be a promising approach for tendon/ligament (T/L) regeneration.[6,14,16](24) Biodegradability is required so that the scaffold can be replaced by native ECM secreted by the cells.[17] Additionally these scaffolds can be used as a substrate for regenerative medicine, drug delivery and implants.[14] To properly design biomaterials, understanding of target functionality and structural stability in addition to host- foreign material compatibility and tolerance is necessary to minimize aseptic inflammation, tissue adhesion and fibrosis of surrounding tissues and to avoid secondary surgical removal.[13,20,21] Beside all the conventional material used for T/L tissue engineering some novel biomaterials can be utilized for better regeneration.

Chitosan: In one study it was found that chitosan a linear polysaccharide has a similar structure to GAG present in the ECM and is biodegradable, biocompatible, anti-bacterial when used as a biomaterial in implantable scaffolds for tendon tissue engineering (Figure 1A).[13] This composite was then seeded by TSPCs in vitro and displayed higher levels of tendon-related gene expression

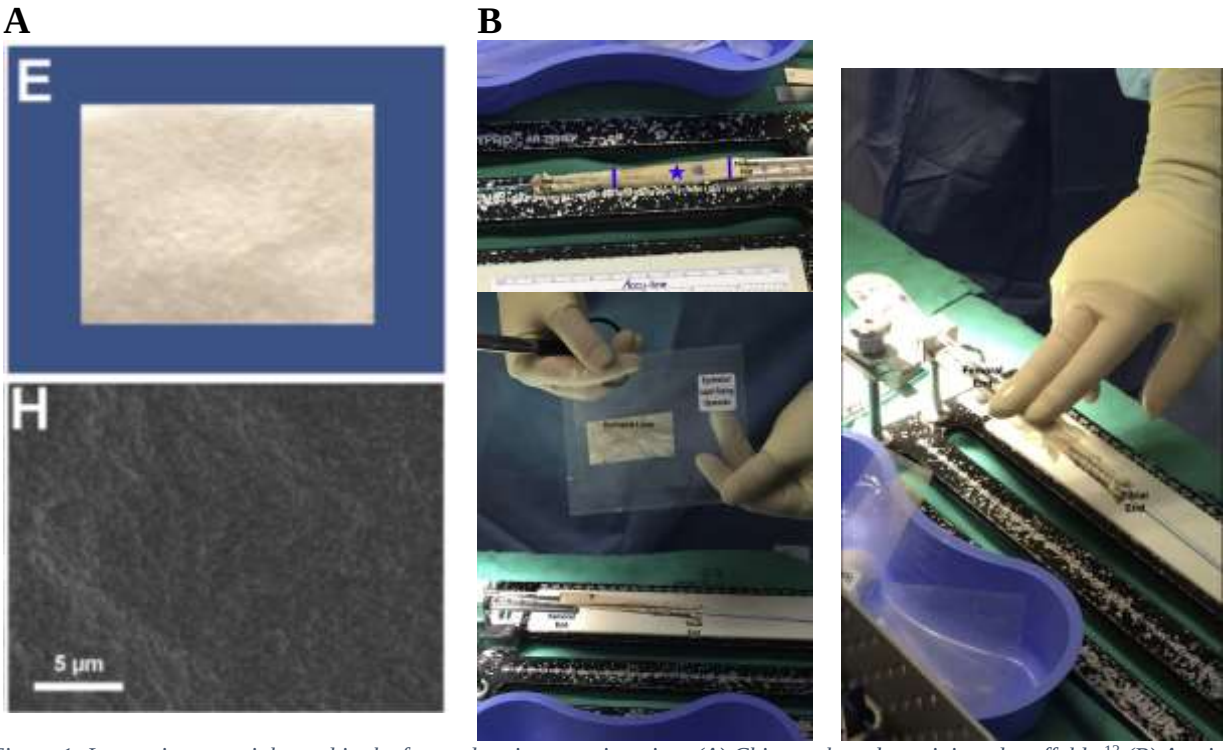

Figure 1: Innovative materials used in the for tendon tissue engineering: (A) Chitosan based precipitated scaffolds;[13] (B) Amnion membrane utilization as an scaffold fabrication biomaterial.[22]

and protein, collagen I (COL1) and collagen III (COL3) production. The chitosan scaffolds are capable of inducing considerable tenogenic differentiation, enhancing tenomodulin activity and increasing maturity, compared with the normal group.[13] On the other hand, the composite was evaluated in rat Achill and it was exhibited that chitosan via the sirtuin-1 signaling pathway can prevent tendon adhesion.[13]

Amnion membrane: Implantation of an amnion matrix with a tendon scaffold, especially during ACL reconstruction, significantly improved anti-inflammatory cytokine secretion and cell proliferation.[22] Moreover, amniotic membranes possess antibacterial functionalities and exhibit protective effects on injury sites by utilizing host growth factors, anti-vasculogenic factors and amniotic membranes indicated to enhance the tissue healing process.[23] Amnion is a membrane forming a fluid-filled cavity (the amniotic sac) that envelopes the embryo.[22] It can be loaded on an implantable scaffold to biologically facilitate the healing process, in particular ACL reconstructions (Figure 1B).[22] It has been reported that amniotic membranes control the down-regulation of transforming growth factors causing various effects on tendon tissue healing, including the recruitment of fibroblasts and macrophages to regenerate the injured tissue.[23]

3D printing

3D printing is one of the manufacturing methods with the following advantages: (1) Customization of the structure (layer-by-layer, …); (2) Complex achievable structures; (2) Inhibition of scaffold delamination; (3) Hugely adjustable pore size resulting in better microenvironments; (4) Simultaneous utilization of different materials using multihead printing and therefore combining their advantages (e.g., combining the biomechanical strength of poly(ε-caprolactone) (PCL) and

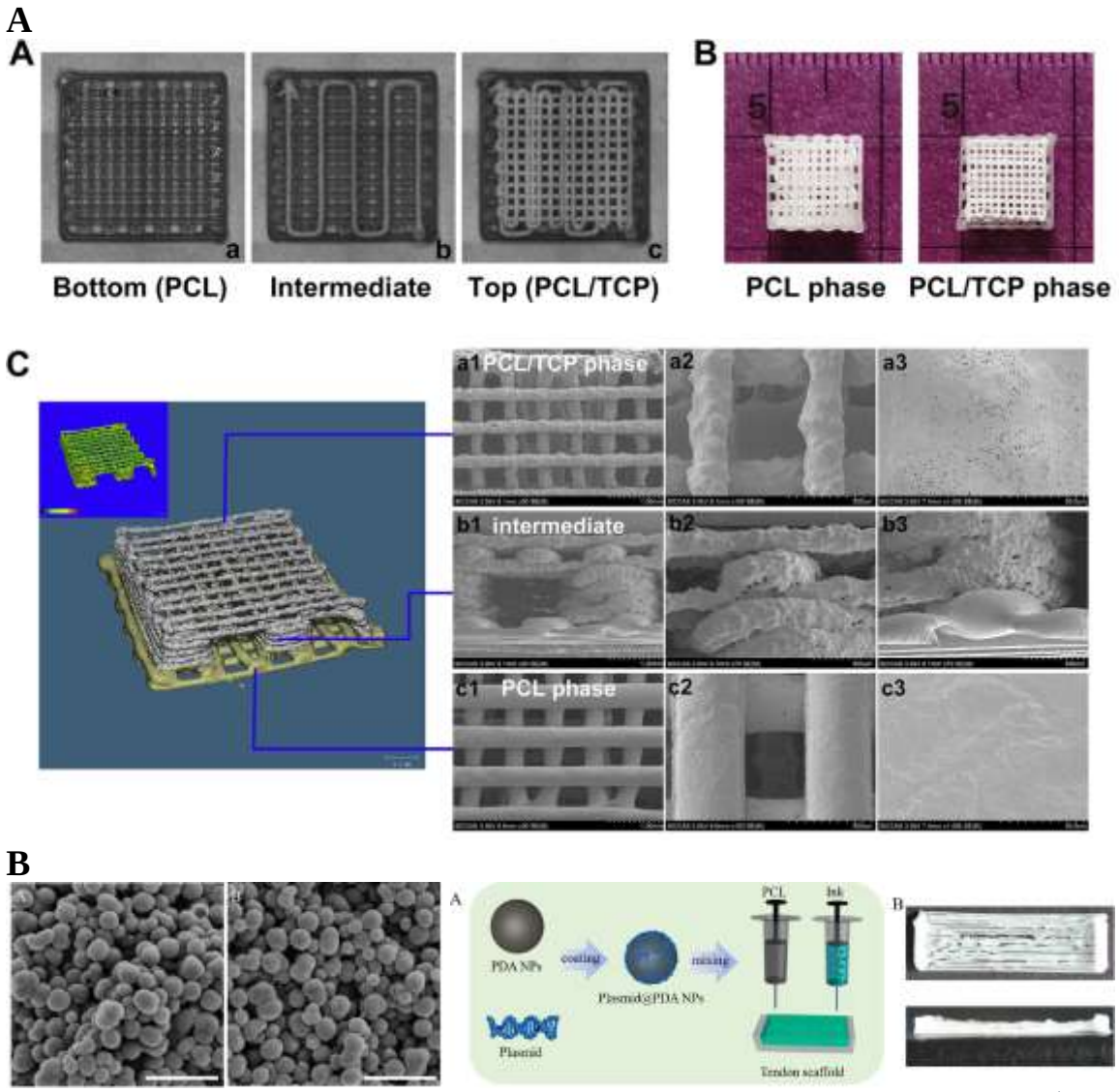

Figure 2: Scaffolds prepared utilizing 3D printing: (A) 3D printed multiphasic PCL–PCL/TCP–PCL/TCP scaffold;[4] (B) 3D printed PCL scaffolds loaded with TGF-β1 gene silencing plasmid: PDA NPs before and after plasmid loading, schematic picture of fabrication method and gross photographs of obtained scaffolds;[12]

the osteoinductivity of tricalcium phosphate (TCP)); (5) Capability of printing cells, molecules and genes.[4,12] But using 3D printing to fabricate scaffolds has the following limitations: (1) 3D printing to print small tissue like scaffolds facing challenges due to the height of the scaffold not being adjustable.[4] A 3D bioprinted multiphasic scaffold was utilized to properly recapitulate the fibrocartilage gradient existing in BTI consisting of PCL–PCL/TCP–PCL/TCP layers representing tendon, fibrocartilage and bone phases respectively (Figure 2A).[4] Tendon adhesions due to fibrovascular scar formation is associated with multiple genes, one of them being transforming growth factor β-1 (TGF-β1), as a subtype of transforming growth factor (TGF).[12] Based on the previous studies showing that the inhibition of TGF-β1 gene expression can promote tissue repair by decreasing tissue adhesion, TGF-β1 silencing plasmid loaded into polydopamine nanoparticles (PDA NPs) loaded into hydrogel were utilized as one of the inks.[12] The external frame of the scaffolds were printed simultaneously utilizing PCL as the other ink to prepare a mechanical

substrate for hydrogels to attach (Figure 2B).[12] These scaffolds promoted tendon regeneration by inhibition of TGF-β1 gene expression when implanted in vivo to heal flexor digitorum profundus tendon defects in a chicken model.[12]

Decellularization

Utilization of synthetic biomaterials to fabricate scaffolds is challenging due to improper recapitulation of the native structure and matrix components and lack of biologically active particles.[5,24,25] Tissue engineered cellular scaffolds exhibit low immunological responses due to the removal of significant amount of cellular components while retaining significant amount of physiological and structural properties and matrix components including collagen, glycosaminglycan (GAG), bFGF and TGF-β1 during decellularization as well as fibronectin with decorin responsible for the mechanical strength of the native scaffolds.[3,5,7,26,27] DNA content change through decellularization can be measured to evaluate cell removal.[25] On the other hand these scaffolds possess a higher amount of collagenous fibrous structure which are responsible for maintenance of mechanical properties and force alleviation during exercise.[7] To obtain acellularized scaffolds, samples undergo physical, chemical and enzymatic treatments.[5,7,28] Freeze-thawing is the most common process between different decellularization strategies, in which cultured cells are subjected to thermal shocks using saline solution and liquid nitrogen multiple times.[26] Book-shaped decellularized scaffolds were developed (Figure 3A) to achieve easier and better decellularization and seeding as a result of faster cell migration from and to the scaffold leading to lower consumption of reagents and higher retainment of the collagenous structure and better removal of the cells and cellular components.[5,28,29] This method was first utilized to decellularize fibrocartilage samples of the pubic symphysis of the New Zealand white rabbits (Figure 3A).[28,29] The obtained results indicated that the fibrocartilage structure remained intact.[28,29] Later on the Achilles tendon of New Zealand white rabbits were decellularized to prepare the scaffolds and then seeded with BMSCs and implanted to heal patella–patellar tendon (PPT) enthesis defect (Figure 3B).[5]

One of the other approaches having the same results is disrupting the endotenon layers by separating the tendon into fiber bundles using lyophilization before decellularization so that the cells are not covered by the endotenon and less chemical reagent will be consumed to remove cellular components.[30] Extensor and flexor tendons of the forelimb or hind limb of adult bovines were decellularized using this method and then implanted into the paraspinal muscle of SD rats.[30] Obtained results indicated that exhibited no significant change of original fibrillar structure and mechanical properties through decellularization and high cell survival in vivo study.[30] The scaffolds promoted the differentiation of the BMSCs into cartilage and tendon.[5] Extracted ECM from human adipose tissue was subjected to hydroxyapatite (HA) gradient to stimulate the calcium gradient present in the BTI and seeded with human UMSCs and then implanted in Sprague-Dawley rats to repair an BTI between the supraspinatus tendon and the bone.[11] The results indicated that unlike other xenogeneic decellularized tissues that induce anti-host inflammation, adipose tissue derived decellularized scaffolds have minimal induction of inflammatory responses probably due to their homogeneity between different species.[11] Utilization of ECM for tendon regeneration was assessed as well in a study with an innovative decellularization method to inhibit tendon

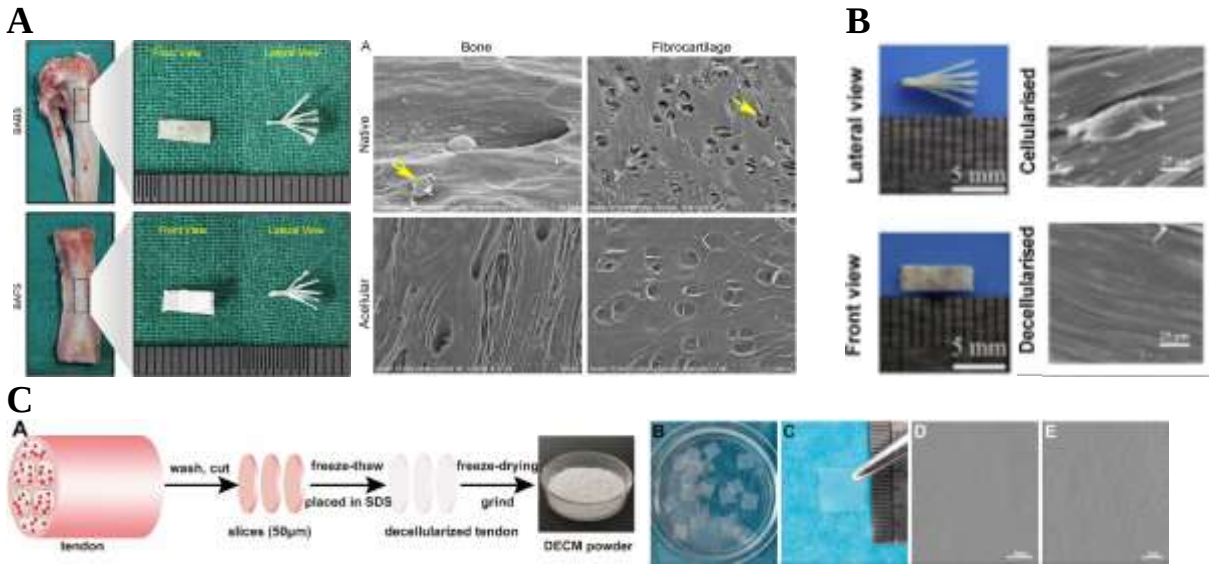

Figure 3: Decellularized scaffolds for tendon/ligament regeneration: (A) Book-shaped decellularized pubic symphysis fibrocartilage samples harvested from New Zealand white rabbits (fresh scaffolds at top and acellularized scaffolds at bottom);[28,29] *(B) Book-shaped decellularized Achilles tendons harvested from New Zealand white rabbits;*[5] *(C) A novel method for decellularization and the obtained decellularized scaffolds: schematic representation of the fabrication method, gross photographs of the wet and dry matrices and SEM images of the wet and dry matrices;*[21]

adhesion.[21] This new decellularization method utilized microsectioning to decrease the amount of time and chemical reagent consumed in decellularization stage and a densely structured physical barrier to prevent tissue adhesion (Figure 3C).[21] Decellularized Achilles tendons harvested from bovine hind legs were implanted in vivo to heal created incisions along the Achilles tendons of New white Zealand rabbits and the results indicated that these decellularized scaffolds could reduce tendon adhesion (Figure 3C).[21]

Electrospinning

The electrospun nanofiber forming a 3D matrix, structurally and mechanically similar to the ECM, can be utilized for T/L regeneration. This versatile and a cost-effective method is important because it will result in a fibrous structure similar to that of the collagenous structure present in the ECM of the native target tissue.[1,9] Although the high surface area of the obtained scaffolds increases the biomolecule-scaffolds affinity but it increases foreign-body reaction (FBR) induction.[10,20] On the other hand fibers obtained from electrospinning can be two orders of magnitude smaller than the ones achieved by other methods resulting in fibers that can mimic the native tissue structure better.[1] The process consists of the following steps: (1) dissolving of the polymer(s) in a solvent (e.g., PCL in HFIP); (2) Spraying the solution under a high voltage; (3) Collecting the fibers using an open-cage or a rotating mandrel after solvent evaporation.[8,20] The electrospun fibers can be knitted or braided to better mimic the hierarchical structure present at tendon ECM.[3,31] Alignment, orientation, pore size and fiber diameter of the obtained nanofibers and other topological factors as well as scaffold surface hydrophilicity can affect inflammatory and immune responses in the host body.[20] It is worth mentioning that aligned fibers recapitulate the structure of the ECM of the tendon consisting of parallel aligned collagen fibrils better than the nonaligned fibers, resulting in better tendon repair.[32,33]In a study, novel coaxial nozzle capable of mid-process adjustment and fabrication of core-shell fibers with the capability of being utilized

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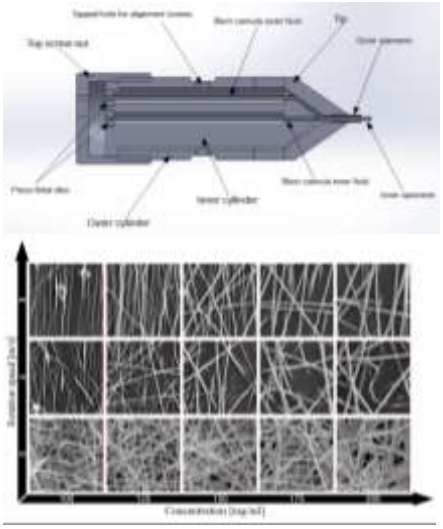

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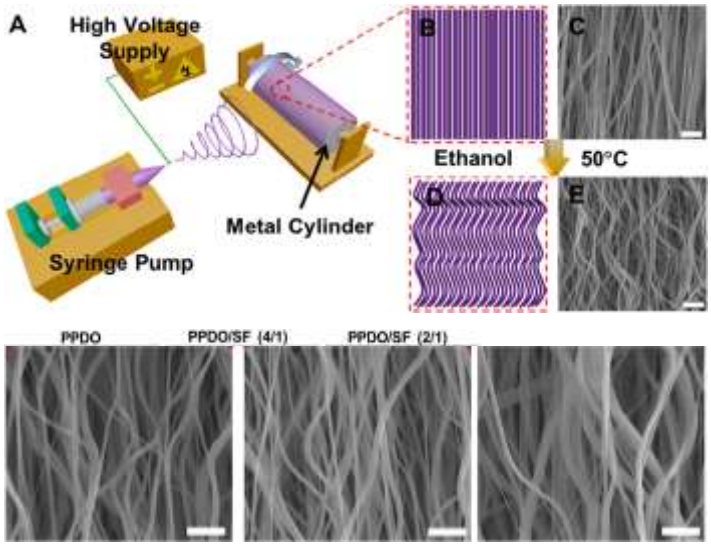
A
High Voltage Supply
Syringe Pump
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C
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PPDO/SF (4/1)
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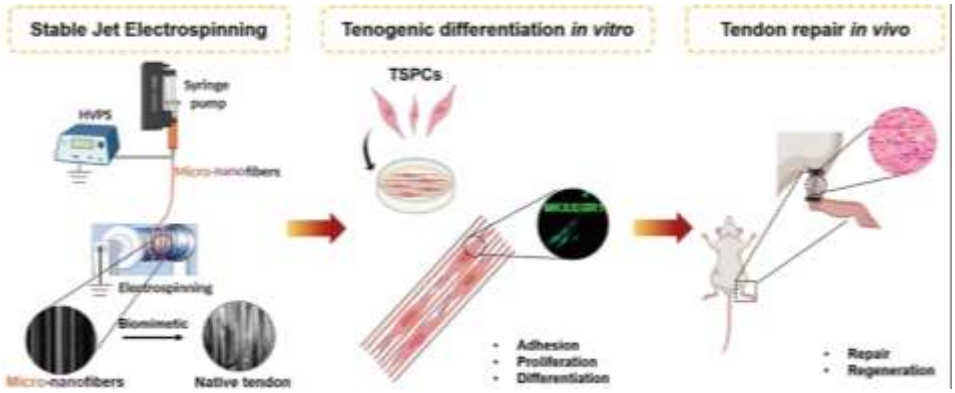
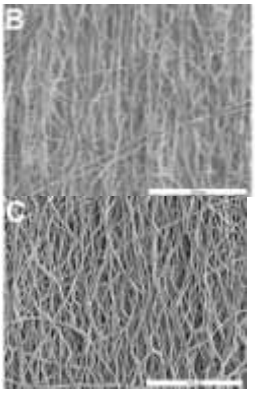

Stable Jet Electrospinning
Tenogenic differentiation in vitro
Tendon repair in vivo
Syringe pump
HVPS
Micro-nanofibers
Electrospinning
Biomimetic
Native tendon
TSPCs
Adhesion
Proliferation
Differentiation
Repair
Regeneration

D


B
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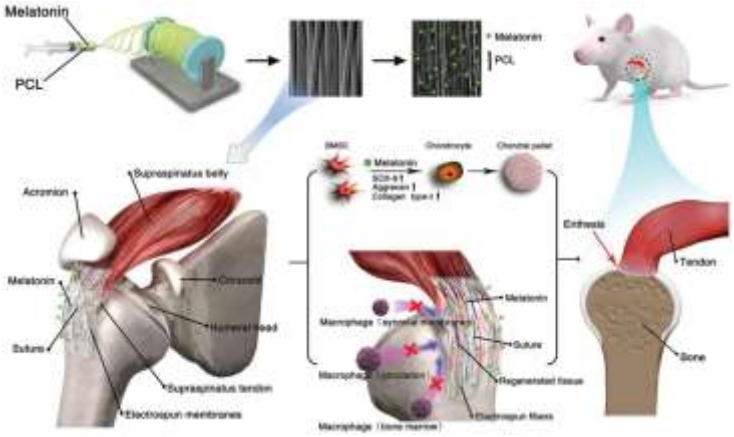
PCL nanofibrous scaffold (bi-layered)
IBP-loaded microspheres
Sintered pocket contained microspheres

F


Melatonin
PCL
Melatonin
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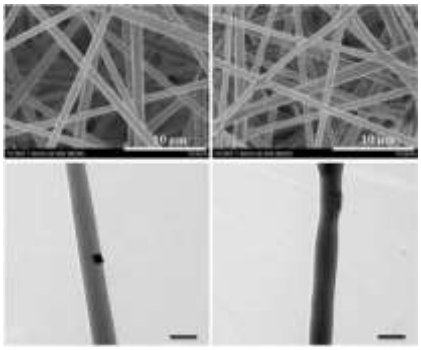
Supraspinatus belly
Acromion
Coracoid
Humeral head
Suture
Supraspinatus tendon
Electrospun membranes
Osteocyte
Chondral pellet
Enthesis
Tendon
Bone
Regenerated tissue
Electrospun fibers

G



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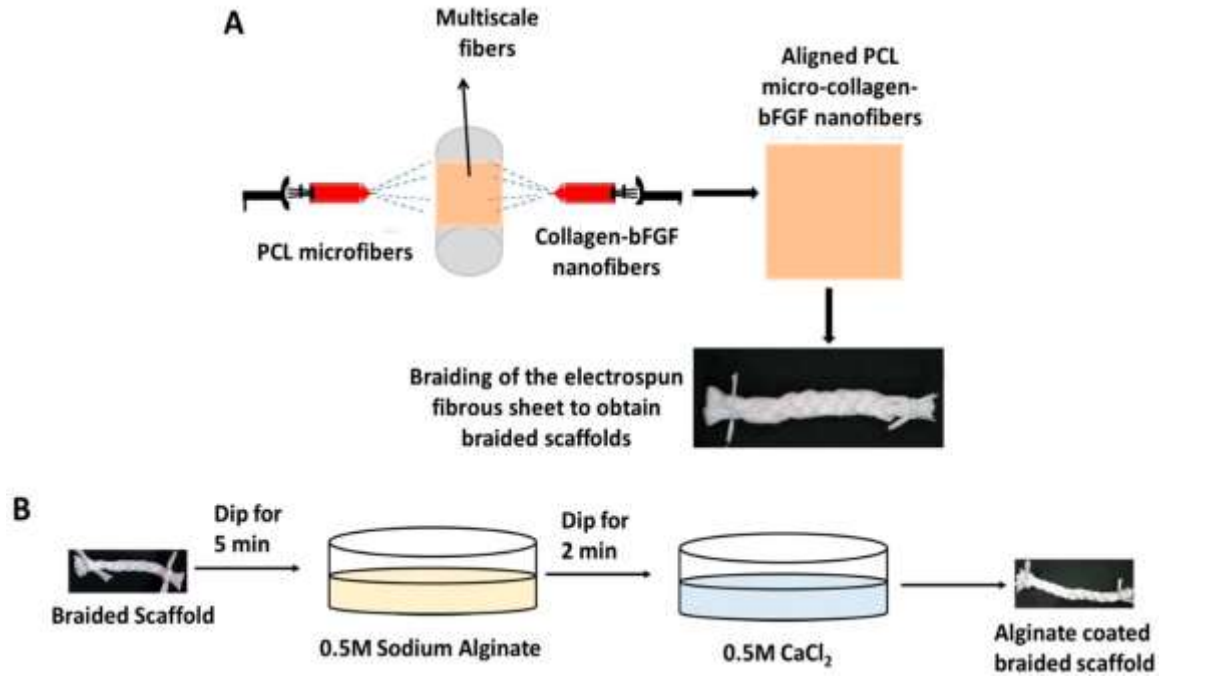

Figure 4: Electrospun scaffolds: (A) PPDO/SF electrospun scaffolds before and after ethanol treatment;[1] (B) Jet electrospun PLLA/PEO scaffolds consisting of micro and nano fibers;[35] (C) SEM images of chitosan free and chitosan loaded PCL aligned nanofibrous electrospun scaffold;[36] (D) PLGA Labrafil modified microspheres loaded with IBP entrapped between two heat sintered electrospun PCL scaffolds: SEM images of the microspheres, top layer and cross section of the microsphere loaded BiLDS;[37] (E) Preparation of melatonin loaded electrospun scaffolds with aligned PCL fibers;[32] (F) SEM and TEM images of the electrospun scaffolds without and with addition of bFGF; [38] (G) Alginate coated braided electrospun PCL and collagen-bFGF fibers;[39]

for drug delivery systems was designed and was used to coaxially extrude two solutions of PCL in TFE with different concentrations on a rotating drum (Figure 4A).[34] Different total concentrations were achieved by adjusting the flow ratio of the inner and the outer flows. The effect of different concentrations and rotation speeds were evaluated.[34] It was shown that higher total concentration led to higher fiber diameter and higher total concentration as well as lower rotation speed led to higher fiber anisotropy (Figure 4A).[34] One of the morphological challenges of this method is to prepare fibers similar to that of the native structure not with a random or aligned microstructure but with a wavy morphology resulting in nonlinear mechanical properties.[1] Poly(p-dioxanone) (PPDO) and silk fibroin (SF) blends with different ratios and pure PPDO solutions in HFIP were electrospun on a cylinder to obtain aligned nanofibrous scaffolds (ANSs) which was immersed into ethanol to obtain nanofibers with wavy morphology (Figure 4B).[1] hTCs cultured with the PPDO/SF fibers exhibited higher proliferation and phenotypic maintenance and a lower inflammatory response than those cultures on pure PPDO scaffolds (Figure 4B).[1] The other morphological challenge of this method is to fabricate scaffolds with anisotropic microfibers and nanofibers (micro-nanofibers) capable of better recapitulation of the native tendon ECM and higher stem cell tenogenic differentiation induction, which can be solved by altering fabrication parameters.[35] In another study, stable jet electrospinning (SJES) of PLLA and PEO solutions in TFE were used to obtain fibers collected by the rotating drum (Figure 4C).[35] Fabrication parameters including solution feeding rate, spinneret tip-to-collector distance and drum rotating

speed were optimally chosen to achieve fibers with specific diameter.[35] The obtained scaffolds were seeded with mouse TSPCs harvested from tail tendon of C57 BL/6 mice and then used to heal a created Achilles tendon defect.[35] The incorporation of the micro and nanofibers resulted in improvement of mechanical properties as well as induction of tenogenic differentiation of the seeded stem cells probably due to better recapitulation of the ECM of the native tissue.[35]

Proper ell attachment necessary for cell survival and utilization of drug delivery systems require high levels of hydrophilicity.[10,37] In a comparative study, a solution of PCL in TFE was electrospun on a rotating drum to obtain orientated fibers and then modified with chitosan and then compared with biocompatible polyurethane-based scaffold (Biomerix 3D Scaffold) to heal created infraspinatus tendon defects in Lewis rat model.[10] It was observed that observed that surface coating did not result in significant change of any of the biomechanical properties and that the biomechanical characteristics obtained from the scaffold did not exhibit any significant defference.[10] In another comparative study, the electrospun PCL scaffolds modified with chitosan and Biomerix 3D scaffolds were implanted subcutaneously in BALB/cJZtm mice (Figure 4D).[36] Improved vascularization and biocompatibility and reduced immune responses could be obsevered after chitosan modification.[36] Non-steroidal inflammatory drugs (NSAIDs) (e.g., ibuprofen (IBP)) are postsurgically consumed drug to mitigate pain and inflammation, however their administration increases the risk of BTI healing failure.[37] However, these detrimental effects are proved to decrease by delayed release of NSAIDs.[37] In another study, Labrafil M 1944 CS oil, a polyethylene glycol (PEG) surfactant, was added to PLGA microspheres to increase their hydrophilicity for drug delivery (Figure 4E).[37] The obtained microspheres were loaded with IBP and buffered in phosphate buffer saline (PBS) were entrapped in between two heat sintered electrospun PCL scaffolds to obtain bilayer delivery system (BiLDS), which was then used to replace detached bilateral supraspinatus of adult Sprague-Dawley rats (Figure 4D).[37] The results indicated that although this scaffold mitigated postsurgical inflammatory response, no significant improvement of biomechanical properties could be observed.[37] One of the other molecules exhibits anti-inflammatory, anti-oxidative and immunomodulatory effects is melatonin.[32] In another study, melatonin dissolved in dimethyl sulfoxide and PCL in TCL were added to obtain a blend solution which was electrospun on a rotating drum to obtain aligned nanofibers.[32] Chondrogenic differentiation of hBMSCs significantly increased by addition of melatonin in vitro and macrophage infiltration was inhibited at the surgery site which led to chondroid zone formation, promoting collagen maturation, decreasing fibrovascular tissue formation and higher biomechanical strength of the regenerated bilateral transosseous supraspinatus tendons of Sprague-Dawley rats (Figure 4F).[32] Other study assessed the feasibility of bFGF utilization in a PLGA electrospun scaffold and its effects on supraspinatus tendon healing in Sprague-Dawley rats in which it was found out that bFGF utilization can improve healing by increasing fibrocartilage and collagen organization formation (Figure 4G).[38] In a study, double stacked braided scaffolds was fabricated by electrospinning PCL and Collagen-bFGF that bFGF (basic fibroblast growth factor) on a collector and coated with alginate to prevent tissue adhesion (Figure 4H).[39] This novel design promotes fibroblast proliferation and enhances expression of tenogenic markers.[39] In a recent study continuous fabrication of electrospun filaments and yarns was achieved and its feasibility for tendon and ligament tissue engineering was assessed.[40] This device consisted of a wire collector including a feeding unit, a wiping unit and a winding unit and an electrospinning unit and motors.[40]

PDO multi-filament yarns obtained using this device promoted healing od infraspinatus incisions in Lewis rats.[40]

Synergistic incorporation of multiple scaffolds

Incorporation of silk fibroin and collagen matrix: Collagen a biodegradable ECM component, promoting neoligament tissue ingrowth, can be utilized as gel and sponge scaffolds for T/L regeneration, but its application is limited due to lack of mechanical strength.[6,7] Knitted SF scaffolds providing internal connective space for collagen matrix can be used to increase the mechanical strength of the obtained scaffolds.[6] Freeze-drying sericin-extracted knitted SF scaffolds immersed in an acidic type I collagen solution under vacuum resulted in collagen microsponge formation which was crosslinked by dehydrothermal treatment (Figure 5A).[6,15] This fabrication method was further evaluated to promote the healing of the created medial collateral ligament (MCL) and anterior cruciate ligament (ACL) incisions in a New Zealand White rabbit model.[6,15] In MCL regeneration study, structural and functional restoration was significantly improved as a result of gene expression and collagen fibril formation regulation.[6] In ACL regeneration study, ACL repair and regeneration was facilitated and short and long-term osteoarthritis (OA) were inhibited.[15] In another study, these scaffolds were seeded with genetically engineered hESC-MSC cells exhibiting high scleraxis (SCX) expression and then evaluated in vivo in a rat Achilles tendon repair model.[41] The results indicated that this scaffold can promote the regeneration of the defect.[41]

Incorporation of Fe_2O_3 nanoparticles and starch/PCL fiber: Stimulation of implanted scaffolds to improve the function of tendon repairing has been presented in a study, that exhibited abundant collagen regarding histological experiments.[42] Magnetic response affected several outcomes in tissue reconstruction, in particular, tenogenic differentiation, which is considered to cause the synthesis of Tenascin-C and collagen type I. In the mentioned study, aligned scaffolds with starch and PCL were fabricated using rapid prototyping, then iron oxide was combined homogeneously into the 3D structure and created a magnetic feature in the composite (Figure 5B).[42] Furthermore, AMSCs are tagged, tracked and activated by MNP (magnetic nanoparticles) for targeted actions such as cell migration and proliferation.[42] In vitro biocompatibility assessment and in vivo tests on rat models indicated the significant effect of structural features and appropriate biochemomechanical stimulations on tendon tissue engineering.[42]

Shell-core scaffolds

Utilizing anisotropic scaffolds can provide more desirable mechanotransduction for tenocytes to form tendon-like fibers. Bioresorbable PCL scaffold consisting of two portions, core and shell, in which longitudinally aligned electrospun nanofibers form the core which is wrapped in a single-layer PCL/PEO film perforated by through holes (Figure 6A). This scaffold creating a conducive microenvironment for cell growth due to the available space for cell adhesion, provides cells with similar alignment to that of the native tissue.[43] Such implantable scaffolds used for tendon tissue regeneration must simultaneously possess optimal mechanical performance, suitable porous structure and biocompatible environment. The core component was obtained by tape-casting an evaporative process, employed to uniformly distribute the CBE (collagen-BDDGE-elastin) gel,

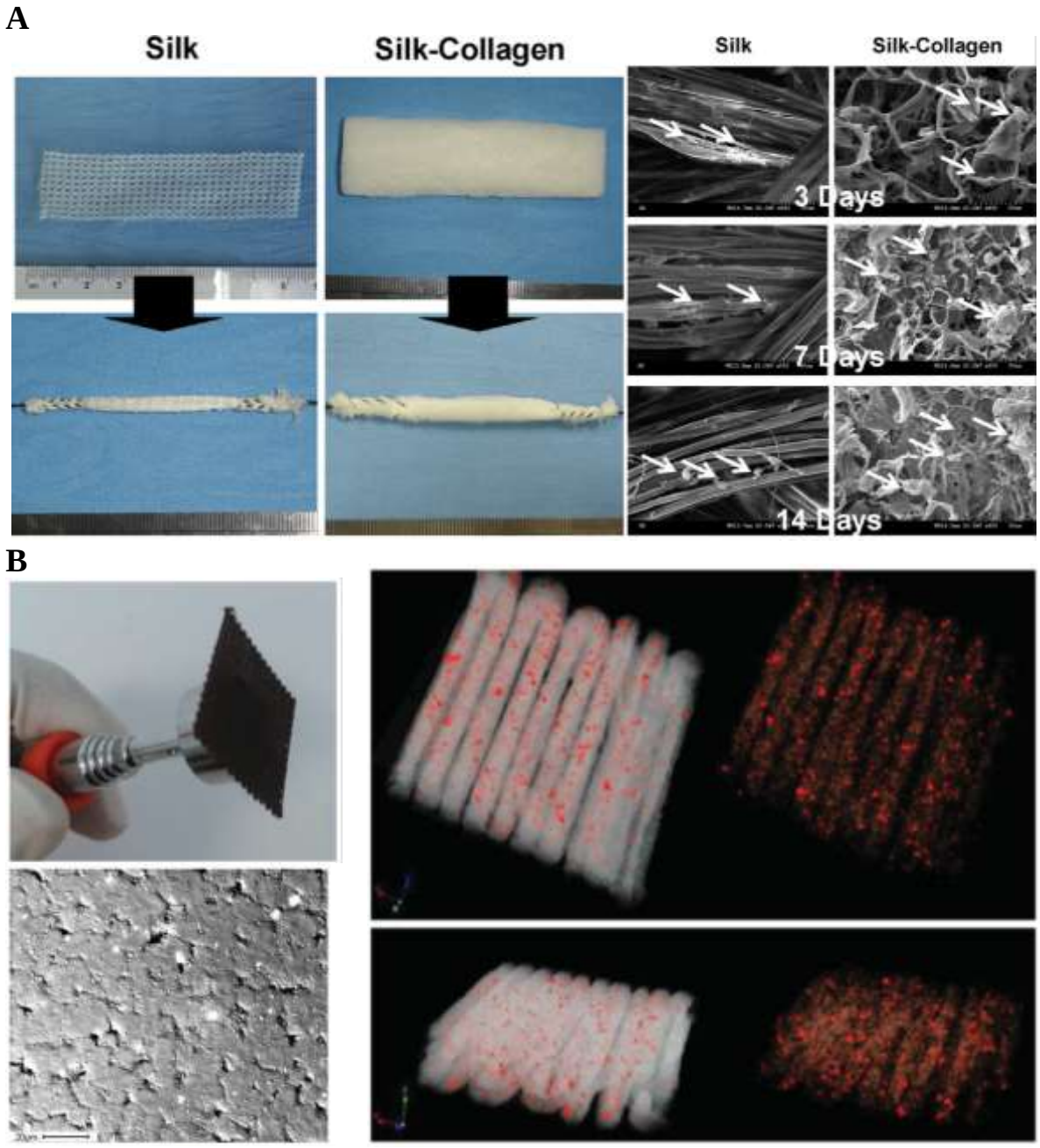

Figure 5: Synergistic incorporation of multiple scaffolds: (A) Synergistic incorporation of knitted SF scaffolds and collagen microsponge;[15] *(B) Starch/PCL nanofiber loaded with* Fe_2O_3 *nanoparticles;*[42]

followed by air-drying to attach the CBE stripes and increase cell density, enhance the scaffold tensile strength and elasticity and increase mechanical strength (Figure 6B).[44] On the other hand, the shell component was shaped as a hollow cylinder surrounding the core and having a highly interconnected porosity promoting cell migration (Figure 6B).[44] The shell is fabricated by uniaxial freezing technique coupled with the typical freeze-drying process to remove the frozen solvent.[44] It is worth mentioning that in this technique pore sizes and pore orientations are widely adjustable by manipulating freezing rate, temperature gradient and collagen concentration of the scaffold.[44]

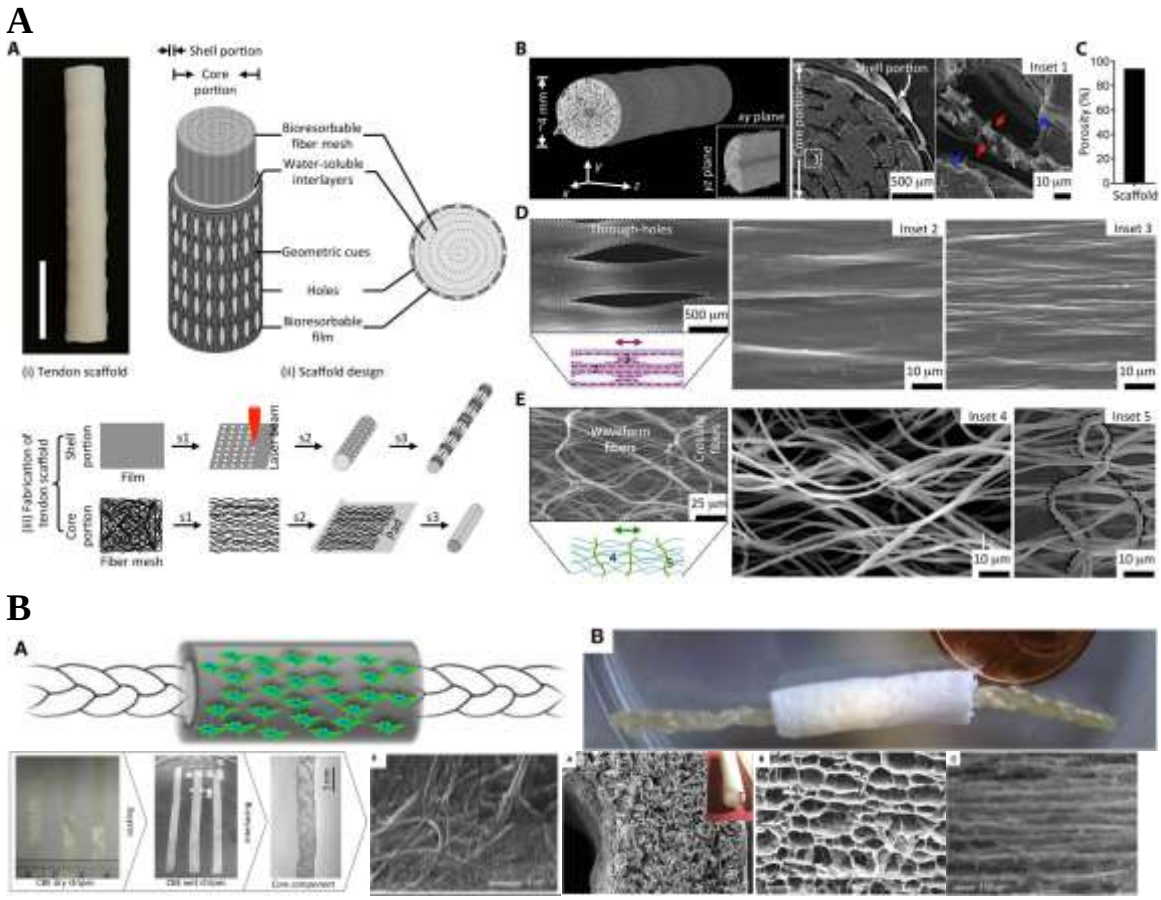

Figure 6: Sell-core scaffolds: (A) Consisting of electrospun PCL nanofibers as the inner portion and perforated PCL/PEO film as the outer portion;[43] (B) Consisting of attached CBE stripes as the inner portion and freeze-dried PTFE as the outer portion.[44]

Knitting

Warp knitted scaffolds obtained using knitting machines can be utilized to generate T/L tissue engineered scaffolds.[6] However, connective neoligament tissues ingrowth is reduced due to their limited internal space of braided or twisted fiber scaffolds.[3] Sometimes further modification of the scaffolds is necessary.[6] Usually knitted silk fibron (SF) can be utilized for tendon tissue regeneration after being subjected to sericin extraction (Figure 7A).[6] This is due to the fact that silk as a natural polymer has high mechanical strength similar to those observed in human ligaments and tendons.[45] Silk is being widely used for high strength tendons (e.g., ACL) based on the fact other synthetic or natural polymers cannot recapitulate the T/L tissue biomechanically.[46]

Melt spinning

In this method melted biomaterial extrusion through a nozzle is used to prepare fibers.[14] Type I rat tail collagen gels loaded with Achilles tendon tenocytes of SpragueeDawley rats were injected into PHBHHx melt spun scaffolds to heal the created Achilles tendon incisions of the rats.[14] The final structure consisted of three PHBHHx fibres passed through the lumen of the PHBHHx porous tube filled with tenocytes loaded collagen gels (Figure 7B) which promoted cellular infiltration, proliferation and cellular alignment/organisation in vivo and in vitro.[14]

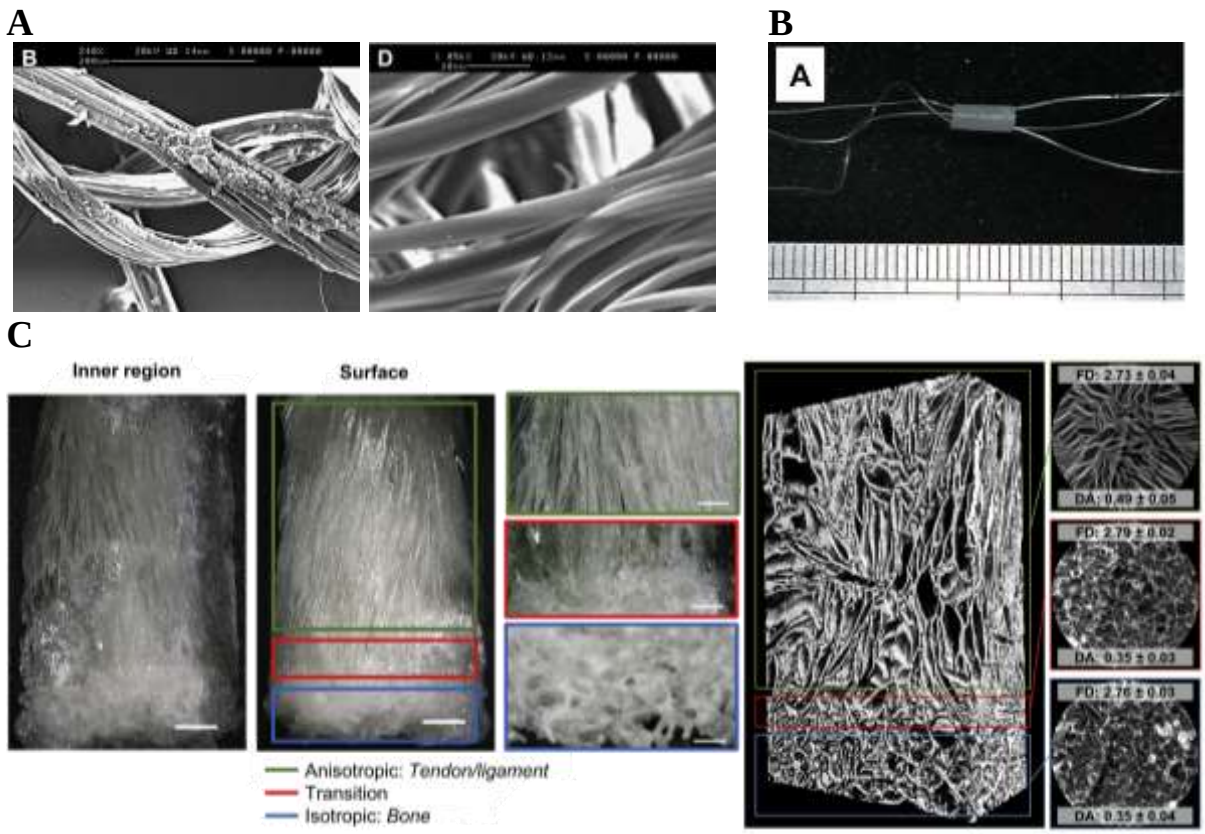

Figure 7: (A) SEM images of knitted SF scaffolds before and after sericin extraction;[6] (B) Assembled PHBHHx melt spun scaffold composed of three fibers passed through the lumen of the porous tube;[14] (C) Platelet rich plasma scaffold; [47]

Platelet rich plasma scaffolds

Many studies were conducted to improve tendon healing by increasing the growth factor density at injury sites and promote vascularization of peripheral tissues. However, growth factor therapies have been limited by the short half-lives of growth factors.[45] There are safety and cost concerns regarding to growth factor release. PRP (Platelet rich plasma) is a method that releases growth factors which contain cytokines and bioactive factors inhibiting inflammation and promoting angiogenesis, ECM augmentation and tissue regeneration (Figure 7C).[47] The use of PRP in orthopedics is still controversial and under consideration and its effectiveness should be further evaluated.

Surface modification

Growth factors promoting cell viability, proliferation and differentiation are a necessary part of cell cultures during cell pre-surgical culturing.[9] Mechano growth factor (MGF) can not only promote T/L repair by promotion of cell proliferation, migration and stem cell differentiation but also regulate inflammatory responses in vitro and in vivo.[20] Silk fibroin (SF) layer-by-layer (LbL) assembly capsules can be utilized for surface modification resulting in to save the activity of biological agents against harsh environmental conditions.[20] SF LbL capsules obtained through purification of silk moth cocoons followed by addition of a set of chemical reagents can be added to the surface of the scaffold in the form of crystalline b-sheet structure after immersion of the SF-

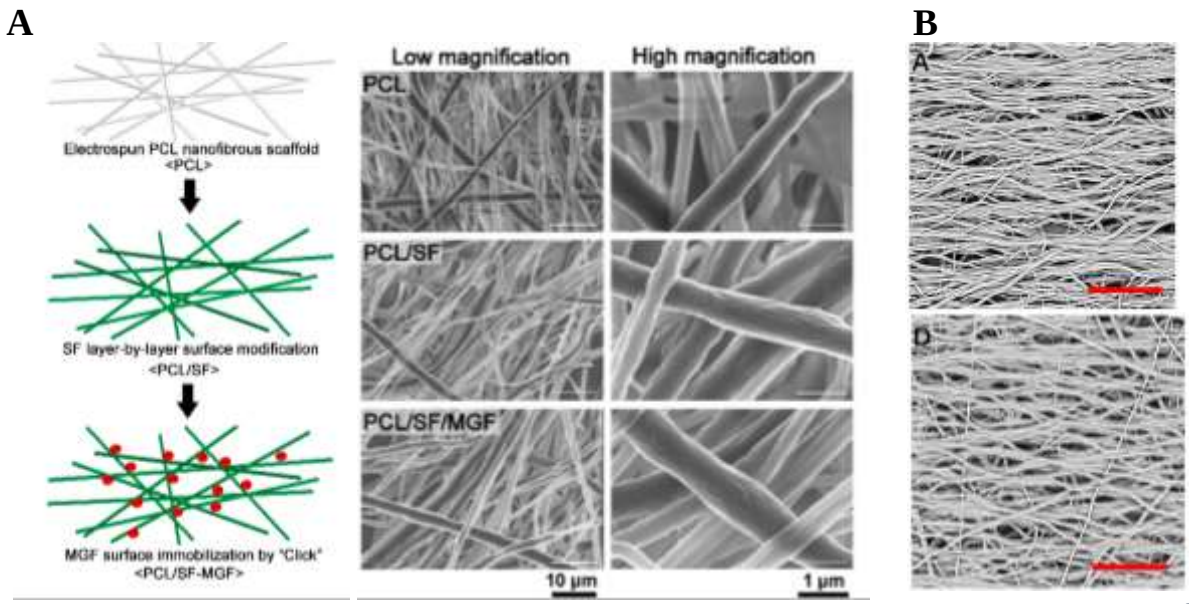

Figure 8: Scaffold modification: (A) Silk surface-functionalization and MGF modification of electrospun PCL scaffolds;[20] (B) Electrospun PCL/Gel aligned nanofibers before and after addition of BP;[9]

coated nanofibers in methanol.[20] Click chemistry can be utilized to fabricate biomolecule/polymer bioconjugation (e.g., bioconjugation of SF MGF) resulting in high accuracy and controllability.[20] Silk surface-functionalized electrospun PCL scaffolds modified with MGF by CuAAC click chemistry were utilized to study the MGF induced modulation of macrophage polarization mechanism and the effect of MGF modification on post-surgical tissue adhesion (Figure 8A).[20] The results indicated that post-surgical tissue adhesion and foreign-body reaction was reduced by MGF induced macrophages transformation into anti-inflammation M2 phenotype.[20,48] Vascular endothelial growth factor (VEGF) a protein necessary for neovascularization exhibits short half-life leading to additional side effects.[9] To overcome this issue, binding peptide (BP) with DRVQRQTTTVVA sequence capable of enhancing exogenous and endogenous angiogenesis of VEGF was added to the surface of the aligned nanofibrous PCL/Gel scaffolds and further investigated to heal patellar ligaments defects of Sprague–Dawley rats (Figure 8B).[9] Utilization of the abovementioned BP promoted regeneration of ligament tissues and formation of the fibrous collagenous matrix.[9] In another study on growth factor release, a novel scaffold was designed, which reduced initial burst release of rhBMP-2 (human bone morphogenic protein 2) leading to enhancement of bone regeneration.[49] It was indicates as well that PLGA/Col microspheres encapsulating rhBMP-2 added to 3D printed PFF (poly-propylene-fumarate) scaffold were able to significantly decrease burst release regarding to collagen delivery and retained its bioactivity for approximately two months.[49]

Cell seeding

Generally, the aligned fibrous scaffolds alone are not sufficient, resulting in homogeneous thin collagen fibers and weak mechanical properties leading to low quality regeneration of the targetted tissue.[50] Biological scaffolds providing seeded cells with a 3D space to grow and proliferate while being stimulated mechanically or chemically to promote tenogenic differentiation seem to be a promising way to restore the biological activity and mechanical properties of the damaged

tendons.[8,45] The bioscaffolds used for cell seeding should be structurally and mechanically similar to ECM of the targeted tissue to regulate cellular activities including attachment, proliferation and differentiation in a way similar to that of the native tissue.[7]

Cell source

One of the challenges of this method is finding proper cell source.[8] The following cell sources can be utilized for cell seeding with the last source being the best one: (1) Irreversibly differentiated cells; (2) multipotent stem cells; (3) coculture of (1) and (2).[4,11]Irreversibly differentiated cells including tenocytes, chondrocytes and osteocytes are irreversibly differentiated cells that have lower metabolism and expression of related markers.[11,51] Tenocytic cells the main cells of the tendon tissues are one of the widely used cells as cell source though their application is limited due to their limited number and high risk off donor site morbidity.[1] Stem cell-based strategies are promising methods in tendon tissue regeneration.[50] Bone marrow-derived mesenchymal stem cells (BMSCs) capable of being differentiated into bone, cartilage and tendon under proper induction can be utilized for T/L regeneration.[4,5,31,52,53] Adipose-derived mesenchymal stem cells (AMSCs) can be harvester and expanded in an easier way and will result in less osteogenic tendency and lower immunogenicity than BMSCs.[31,53,54] Umbilical cord-derived mesenchymal stem cells (UMSCs) exhibit faster proliferation, less change by aging, lower immunological responses in comparison to the other MSCs.[11,31,53] Proliferation and function of many inflammatory immune cells, including T cells, natural killer cells, B cells, monocytes, macrophages and dendritic cells, when restricted by MSCs, stimulate the transition of TH1 to TH2 cells, upgrade tissue regeneration and create a shift from macrophages M1 to M2, pro-inflammatory to anti-inflammatory, respectively.[31] Tendon stem progenitor cells (TSPCs) can proliferate faster and have higher clonogenicity and multi-differentiation potential than BMSCs.[7] Tendon-derived stem cells (TDSCs) capable of colony formation, self-duplication, multi-directional differentiation show higher multi-directional differentiation, self-renewal capacity more tenogenic differentiation and related mRNA expression than the MSCs.[8,55] Embryonic stem cells (ESCs) van be induced to improve T/L regeneration.[41] For cell seeding, the autologous or allogeneic cells can be collected and seeded onto the composite scaffolds uniformly to form cell-scaffold constructs and then kept in an incubator.[56] Afterwards, cell-seeded scaffolds are coated with cross-linking agents such as sodium hyaluronate or glutaraldehyde (GA).[56] A study was conducted to compare cell-free, allogenic and autologous cells, seeded on scaffolds.[56] The results demonstrated that mechanically stimulated cells of all the three groups could achieve similar tendon tissue regenerations in vitro and in vivo , but autologous cells possessed slightly thicker collagen fibers and better degradation rates.[56] It is essential to evaluate the inuced differentiation of stem cells via a topography-based framework on to enhance the orientation and differentiation of stem cells simultaneously.[57] Age of the stell cell donors may affect the obtained results due to the fact that occurrence of tendinopathies and ECM composition and cyoskeletal turnover changes by age.[58] Expression of tendon–related genes (tenomodulin, alpha-1 collagen type I, Sox-9 and Aggrecan) can be measured using quantitative real time - polymerase chain reaction (qRT-PCR) to quantitively evaluate the differentiation of the stem cells into T/L.[5,57] Stromal cell-derived factor-1a (SDF-1a) could improve tendon regeneration by recruiting endogenous cells.[60] There was an innovative approach in one study, SDF-1a can attach collagen scaffold surface via the collagen-binding

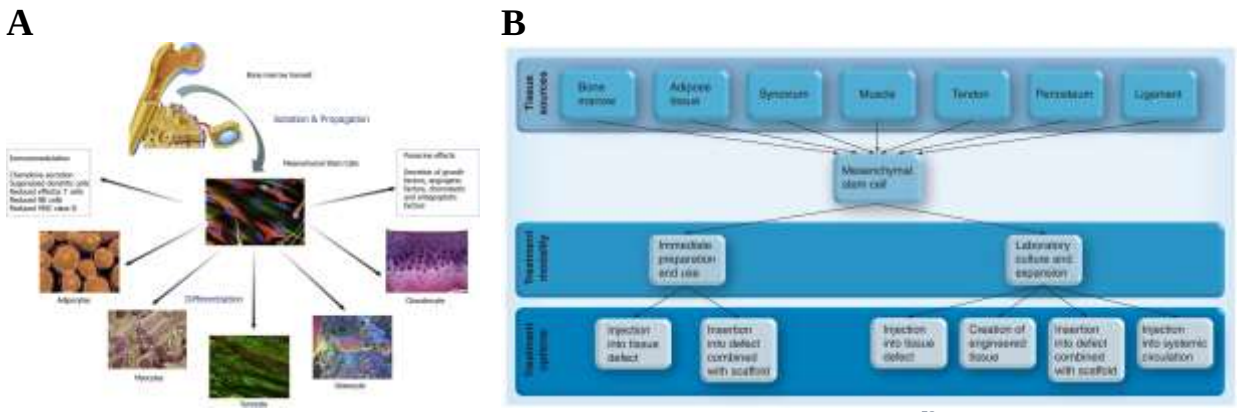

Figure 9: (A) Bone marrow-derived mesenchymal stem cells (BMSCs) harvest and differentiation;[52] (B) Potential sources to harvest MCSCs and their applications in treatment.[59]

domain that can improve the migration of mesenchymal cell, dermal and Achilles tendon fibroblast. SDF-1a can promote the recruitment of CXCR4+ fibroblast-like cells followed by Tenascin-C deposition, which is necessary for tendon repair development.[60]

Static seeding

Static seeding can result in faces challenges in multiple cell type seeding with low cell capacity in the intermediate phases and uneven distribution.[4,5] In a comparative study, decellularized porcine tendon, bone and dermis tissues seeded with human TSPCs harvested from Achilles tendon samples were utilized to treat created Achilles tendon defects in a nude mouse model.[7] The obtained results indicated that although stem cells could adhere and proliferate in all the specimen, they affect stem cell fate with tendon-derived decellularized scaffold exhibiting the highest tenogenic-lineage differentiation and lowest osteogenic-lineage differentiation (Figure 10A).[7]

Cell loaded hydrogel

Multiple cell types can be loaded into hydrogels and then attached to the scaffold.[1] in vitro and in vivo studies exhibited printed cells encapsulated by hydrogels utilized for tissue engineering could survive and promote tissue repair.[12] Gelatin methacrylate (GelMA) a cell responsive hydrogel can be utilized for cell encapsulation.[1] GelMA containing FBs, BMSCs and OBs were used to regenerate teno-, fibrocartilage- and osteo-like tissues in BTI healing.[1] Crosslinking GelMA can be performed by visible light radiation (Figure 10B) and due to its short time of light exposure its influence on cell viability will be minor.[1,61] In another study, fibrin hydrogel obtained from human fibrinogen encapsulating AMSCs were loaded into a electrospun PLGA and implanted in Sprague-Dawley male rats to repair a supraspinatus tendon defect.[62]

Cell sheets

These sheets can be obtained by culturing MSCs on temperature-responsive culture dish followed by placing the culture dish at room temperature and then implanted by being physically put between scaffold layers.[5,63] Bone marrow mesenchymal stem cells sheets (MSCSs) sandwiched with book-shaped scaffolds were utilized for BTI regeneration resulting in significant improvement of the bone and fibrocartilage generation and better mechanical properties.[5,63]

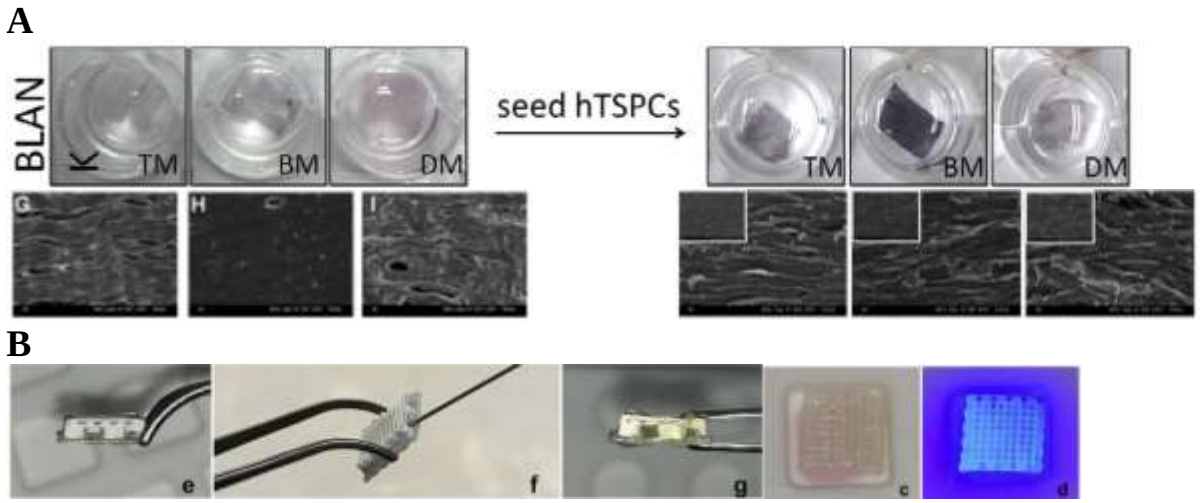

Figure 10: Cell-scaffold constructs: (A) Static loading of hTSPCs on tendon-, bone-, dermis-derived matrices before and after cell seeding;[7] (B) FBs, BMSCs and OBs loaded GelMA injection and photocrosslinking;[4]

Biochemomechanical stimulation

Biochemomechanical cues are necessary for proper stem cell proliferation, migration, differentiation and apoptosis, although due to lack of understanding of T/L development finding suitable cues is a challenge.[4,8] Mechanical stimulation can promote cell proliferation, collagen fibers formation, ECM production which result in improvement of mechanical properties of the tissue engineered constructs.[8] Electrospun poly(L-lactide-co-ε-caprolactone)/collagen P(LLA-CL)/Col scaffold with aligned nanofibers seeded with TDSCs harvested from bilateral patellar tendons of Oryctolagus cuniculus was subjected to dynamic mechanical load and then planted in vivo and in situ for further evaluation (Figure 11A).[8] Obtained results indicated that dynamic mechanical load promotes the expression of tendon-related proteins and maturation of regenerative tendon tissues and enhanced the mechanical properties of the injured tendons.[8] Composite scaffolds composed of an inner part of longitudinally arranged PGA unwoven fibers and an outer part of a knitted PGA and PLA fibers in the form of a net, seeded with AMSCs harvested from nuchal subcutaneous adipose tissue of New Zealand White rabbits were subjected to dynamic stretch in a periodic manner in a bioreactor (Figure 11B).[54] It was observed that implanted cells could produce ECM which resulted in better integration of seeded cells with the scaffold materials.[54]

Growth factors promoting cell viability, proliferation and differentiation are a necessary part of cell cultures during cell pre-surgical culturing.[9] PPDO/SF wavy nanofibrous electrospun scaffolds seeded with human adipose derived mesenchymal stem cells (hADMSCs) were subjected to cyclic stretching stimulation in a bioreactor while being cultured with tenogenesis induction medium to provide the cell with TGFβ3 necessary for differentiation of the cell into tenocytes.[1] It was observed that biochemomechanical stimulation promoted tenogenic differentiation of hADMSCs (Figure 11C).[1] In another study TSCs and BMSCs harvested from the hind limb Achilles, tendons and tibia and femur of Sprague-Dawley (SD) rats were seeded onto decellularized Achilles tendons scaffolds in different ratios and then were subjected to a concentration gradient of osteogenic induction medium created by a polydimethylsiloxane/poly (methyl methacrylate) PDMS/PMMA

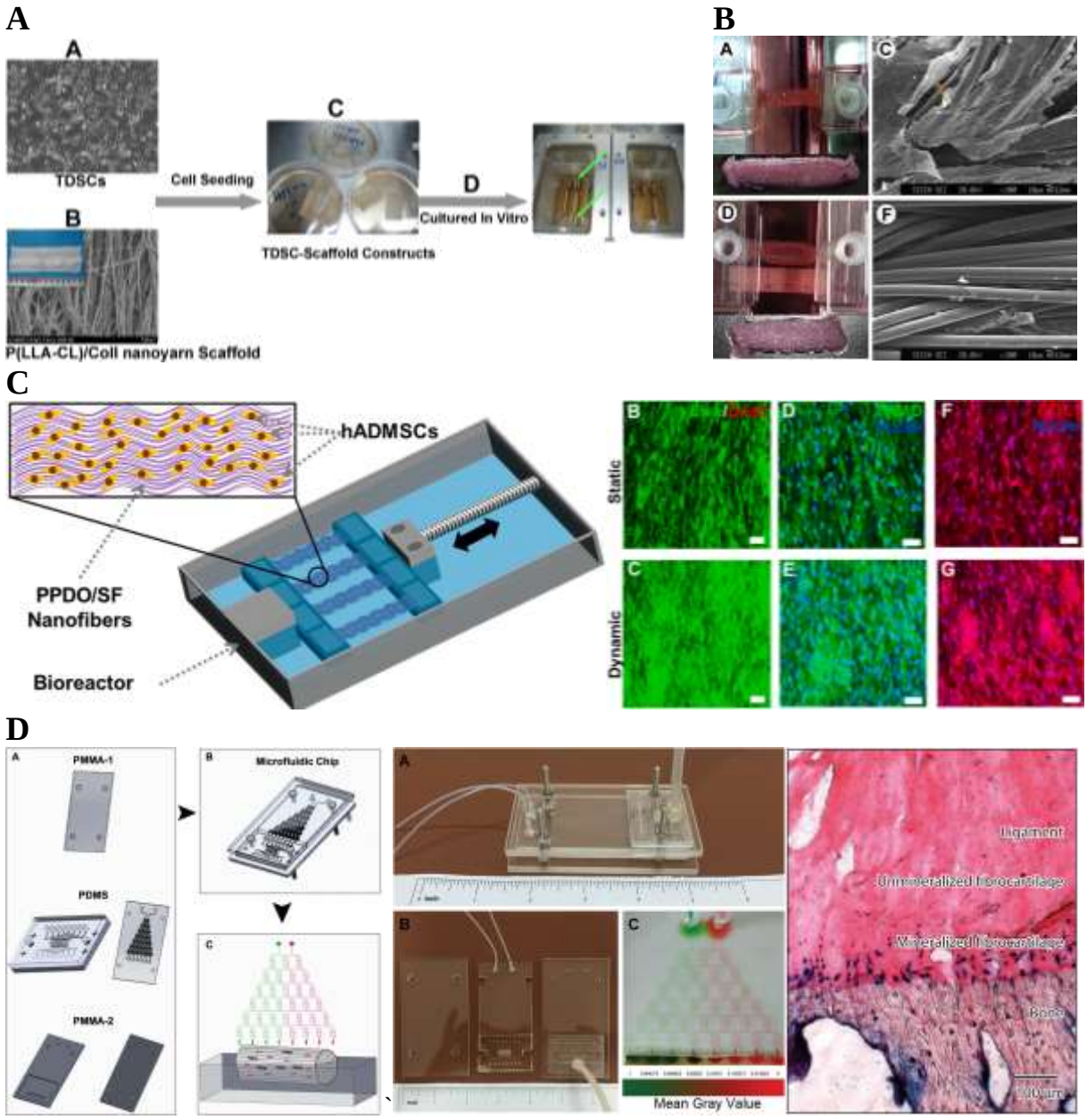

Figure 11: Biochemomechanical stimulation: (A) Mechanical stimulation of the P(LLA-CL)/C scaffold seeded with TDSCs of Oryctolagus cuniculus;[8] *(B) Mechanical stimulation of PGA/PLA hybrid scaffold in a cell-cultured bioreactor (Top cell seeded and bottom cell free);*[54] *(C) Biochemomechanical stimulation of the hADMSCs seeded of PPDO/SF wavy nanofibrous electrospun scaffolds;*[1] *(D) Microfluidic chip designed to create osteogenic induction medium concentration gradient;*[64] *(E) Structure of the enthesis consisting of ligament, unmineralized fibrocartilage, mineralized fibrocartilage and bone.*[65]

chip (Figure 11D) to recapitulate different parts of the enthesis including tendon, decalcified fibrocartilage, calcified fibrocartilage and bone (Figure 11E).[64–66] The obtained cell loaded biochemically stimulated scaffolds were implanted in SD rats for Achilles tendon enthesis repair.[64] The obtained results indicated that this method is feasible for enthesis regeneration.[64] Localized ultrasound-mediated microbubbles enhance the therapeutic gene delivery, which recruits endogenous cells to achieve better ligament bonding. When BMP6 encoding DNA was being delivered using ultrasound, the endogenous mesenchymal progenitor cells were stimulated

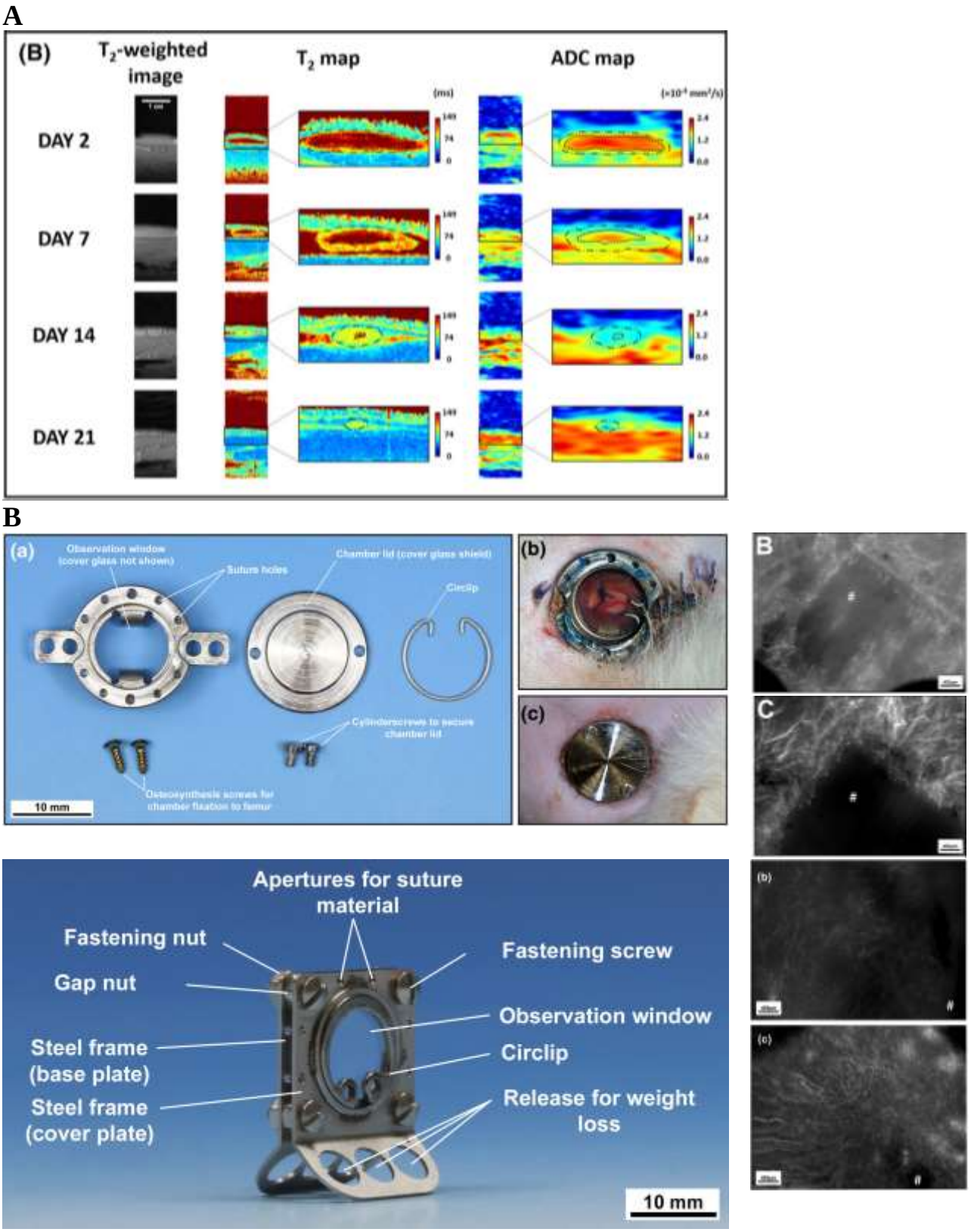

Figure 12: Noninvasive imaging techniques: (A) T_2 and ADC images obtained by MRI; (B) Dorsal skinfold and rat femur chamber used for intravital microscopy imaging of the targeted tissues and the obtained images. [36,67]

genetically which was effective for the improvement of ligament reconstruction.[68] However, prolonged ultrasound exposure can exhibit adverse effects such as unreasonable tissue warm-up and dystrophic calcifications leading to tissue lesions.[68]

Noninvasive imaging techniques

One of the important parts of evaluating a suitable scaffold, usually performed in most studies is the histological assessments.[69] In vivo evaluation of biomaterials and implanted composites is performed by sacrificing the animals to obtain the regenerated tissue.[69] Present methods for scoring scaffold regeneration are based on pre-established semi-quantitative scales, such as vascular and tissue internal growth.[69] As we know, pathologists score the tissue sections based on a pre-established range of parameters.[57] These parameters including proliferation and differentiation of cells, the external body feedback, the neo-vascularization among the implant and the sphere of tissue surrounding the composite implant have to be evaluated.[69]

MRI: MRI, a non-invasive monitoring technique, reduces the need for scaffold harvesting and calculates T2 relaxation time and apparent diffusion coefficient (ADC) combined with standard histological evaluation (Figure 12A).[69,70] Results of our findings indicate that MRI can be used as a non-invasive technique to assess the regeneration of injured tissues by evaluating the cellular infiltration, void area fraction and angiogenesis in collagen scaffolds.[69] These characteristics can indicate if the regeneration quality was acceptable or not.[69]

Chambers: Chambers with observation windows can be implanted to observe the surgery site. Recently they have been installed on the skin near the left hind limb and dorsal skin of Lewis/Han Ztm rats and BALB/cJZtm mice of chitosan modified electrospun PCL scaffolds were fixed on the femur (Figure 12B).[36,67] Intravital microscopy were taken through the window and functional capillary density was evaluated (Figure 12B). [36,67] It was found that chitosan coating increased neovascularization of the targeted tissue neovascularization becomes higher the closer we get to the center of the implant. [36,67]

Conclusion

Tendons and ligaments are connective tissues responsible for mechanical stability and stress transformation. These tissues are being frequently damaged during excessive activities. Low vascularity and cellularity of the abovementioned tissues result in slow and improper regeneration. Surgical fixations utilizing autografts, allografts and xenografts are the traditional techniques for fixing these tissues after injury. Despite the enhancements of the surgical procedures, the biomechanical and histological properties of the regenerated tissues are not restored. Due to the limitations of these surgical procedures, researchers were to develop tissue engineering methods that could overcome these problems. Tissue engineering provides cell-scaffold constructs that can be implanted in injury site for better tissue regeneration. The scaffolds are fabricated using different methods (e.g., 3D bioprinting, electrospinning, decellularization and …). Stem cells and irreversibly differentiated cell obtained from autologous, allogenic and xenogeneic tissues can be loaded on these scaffolds. These cells loaded scaffolds can be subjected to biochemomechanical stimulation to induce their tenogenic differentiation. The obtained cell-scaffolds at this stage can be implanted in the animal models but, its effect on healing process has to be evaluated. These

postsurgical assessments were performed after different time intervals to correlate the regeneration progress and healing time. Some studies were conducted to achieve continuous or semi-continuous observations of the animal models so that the number of the animals sacrificed in the experiments decreases while the frequency of the experiments increases. Based on the acceptable animal study results indicating acceptable regeneration and the similarities between the targeted tissues in those animals and humans, it can be concluded that these tissue engineered grafts are suitable for human tendon and ligament tissue regeneration.

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